Mycobacterial Type VII Secretion Systems From Structure To Function

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2020

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is an ancient pathogen that has coevolved with humanity. DNA that is very closely related to the genetic code of *M. tuberculosis* has been isolated from mummies buried over 9.000 years ago (Hershkovitz *et al.*, 2008). More so, evolutionary studies have determined that members of the *M. tuberculosis* complex (MTBC), a group of mycobacteria that are genetically closely related to *M. tuberculosis*, have emerged approximately 70.000 years ago and have accompanied out of Africa migrations of anatomically modern humans (Comas *et al.*, 2013). Although TB is often considered a disease of the past in developed countries, due to the low number of cases in these areas, the disease is still a big societal problem. In 2018 alone, TB still accounted for over 1.5 million deaths and 10 million new cases worldwide, making *M. tuberculosis* the most successful bacterial human pathogen (WHO, 2019). More so, projections show that approximately a quarter of the world population was infected with latent *M. tuberculosis* in 2014 (Houben & Dodd, 2016).

Despite worldwide commitment through the "End TB" strategy, the number of TB cases and deaths has shown only a marginal decrease. Concurrently, the number of cases that involves multi-drug resistant (MDR) and extreme-drug resistant (XDR) *M. tuberculosis* strains has been rising. WHO estimates indicate that between 2009 and 2014 the number of MDR TB cases has increased three-fold. In 2018, approximately half a million new cases were diagnosed with resistant forms of TB (WHO, 2019). Treatment of drug susceptible TB requires a cocktail of three to four antibiotics, given over the course of approximately six months. When drug resistant strains are detected, alternative treatment regimens are required for up to two years for MDR-TB and four years for XDR-TB strains. Globally, while the rate of treatment success is 85% for drug-susceptible TB, this falls to 56% for MDR-TB and only 39% for XDR-TB (WHO, 2019). Additionally, patient treatment costs skyrocket when drug resistant strains are involved, with costs between ~100.000 and 500.000$ per XDR-TB carrying patient (Diel *et al.*, 2014, Manjelievskaia *et al.*, 2016). The long treatment periods combined with a lack of patient compliance to the treatment feeds into the existing problem of drug resistant TB.

Currently, the only licensed vaccine for prevention of TB disease is the live Bacille Calmette-Guerin (BCG) vaccine, which is based on *Mycobacterium bovis*, a species belonging to the *M. tuberculosis* complex which causes TB-like disease in cattle

and humans (WHO, 2019). It has been developed by Calmette and Guerin in the 1920s, by sub-culturing a virulent strain for 10 years, during which it has accumulated various genetic mutations and deletions. This has resulted in an attenuated strain that is no longer able to cause disease but is still capable of eliciting an immune response. Although the vaccine is able to protect against severe cases of TB in children, it is not effective in preventing TB in adults. Interestingly, BCG has been known to stimulate the immune system aspecifically, which results in increased protection of vaccinated children against other diseases and was the basis for immunotherapy against some forms of bladder cancer (Fuge *et al.*, 2015).

Although *M. tuberculosis* is the most notorious mycobacterial pathogen, it is not the only disease-causing mycobacterial species. Members of the MTBC, including *Mycobacterium africanum*, *Mycobacterium canetti* or *Mycobacterium microti*, cause tuberculosis in humans or other animals. Other important human pathogens of this genus include *Mycobacterium ulcerans* – causing Buruli ulcers, a chronic and debilitating disease of the skin – and *Mycobacterium leprae* – causing leprosy, another ancient disease that is still a health problem in developing countries. A group of mycobacteria, known as non-tuberculous mycobacteria (NTMs), which includes *Mycobacterium avium* and *Mycobacterium abscessus*, are also infectious to humans, with the most common clinical manifestation being lung diseases (Johansen *et al.*, 2020). Moreover, a range of mycobacterial species have an economic impact, by causing diseases in various animal hosts, such as *Mycobacterium bovis* and *Mycobacterium avium paratuberculosis* for cattle (Thoen *et al.*, 1981) and *Mycobacterium marinum* in fish (Decostere *et al.*, 2004).

Mycobacterial pathogens are able to thrive in their niche environments due to a number of virulence factors that characterize these bacilli. One such defining feature is the presence of a highly impermeable cell envelope. This impermeability stems from a distinct outer membrane (OM), which is different in composition than that of Gram-negative bacteria, comprising mainly of specific long chain fatty acids (C60-C90), called mycolic acids (Hoffmann *et al.*, 2008). The mycobacterial outer membrane also contains a large number of other unique lipids, some of which are able to manipulate the immune response. This hydrophobic layer is covalently linked to a periplasmic arabinogalactan-peptidoglycan layer (Dulberger *et al.*, 2020). The highly impermeable OM protects the bacterium from a suite of antibiotics and host defense mechanisms.

The primary site of infection by *M. tuberculosis* is the lungs. When *M. tuberculosis*-containing aerosols reach the lower lung, recruited alveolar macrophages phagocytose the bacteria (Figure 1). Their highly specific cell envelope ensures that these bacteria are able to survive inside these host immune cells. In addition to its special lipids, pathogenic mycobacteria use an array of mechanisms to successfully infect and reside in the human body (Buter *et al.*, 2019, van der Wel *et al.*, 2007, Ates *et al.*, 2016). In particular, virulence factors secreted by a specialized secretion system, called ESX-1, allow the bacterium to escape the phagosome to the more nutrient rich cytosolic environment (van der Wel *et al.*, 2007). New macrophages are recruited at the site of the infected macrophages, giving rise to the early stage of a so-called granuloma. At this stage, further infection can take place through bacteria that infect the newly recruited macrophages. The center of the tuberculous granuloma is a compact and organized aggregation of macrophages that have highly interlinked cell membranes that link adjacent cells (Ramakrishnan, 2012). Additional cell types have been found in granulomas, including B and T cells, neutrophils, dendritic cells (Ramakrishnan, 2012). The late stage granuloma can become encapsulated, which limits bacterial growth. The second option is that late granulomas can become necrotized. In necrotized granulomas, there is widespread cell death at the center, which allows the bacteria to grow out in high numbers and subsequently be transmitted to other hosts (Cambier *et al.*, 2014, Ramakrishnan, 2012). The third possibility is that granulomas remain stable and *M. tuberculosis* can reside asymptomatically for prolonged periods of time in the formed granuloma, aided by its very slow growth rate, by the ability to slow down its metabolism and by its impermeable cell wall. At later time points, bacteria can go out of this so-called dormant or persistent stage and start replicating, reactivating the infection. Current projections estimate that up to one quarter of the world population carries the disease asymptomatically. Collectively, this makes tuberculosis a worldwide threat.

To overcome the highly specific mycobacterial cell envelope for the transport of (macro)molecules, mycobacteria utilize multiple related specialized secretion systems, collectively named type VII secretion systems (T7SSs). First discovered in *M. tuberculosis* in 2003, related T7SSs were later found in several other *Actinobacteria* species and also in *Firmicutes* (Stanley *et al.*, 2003). Mycobacteria encode up to five different T7SSs,

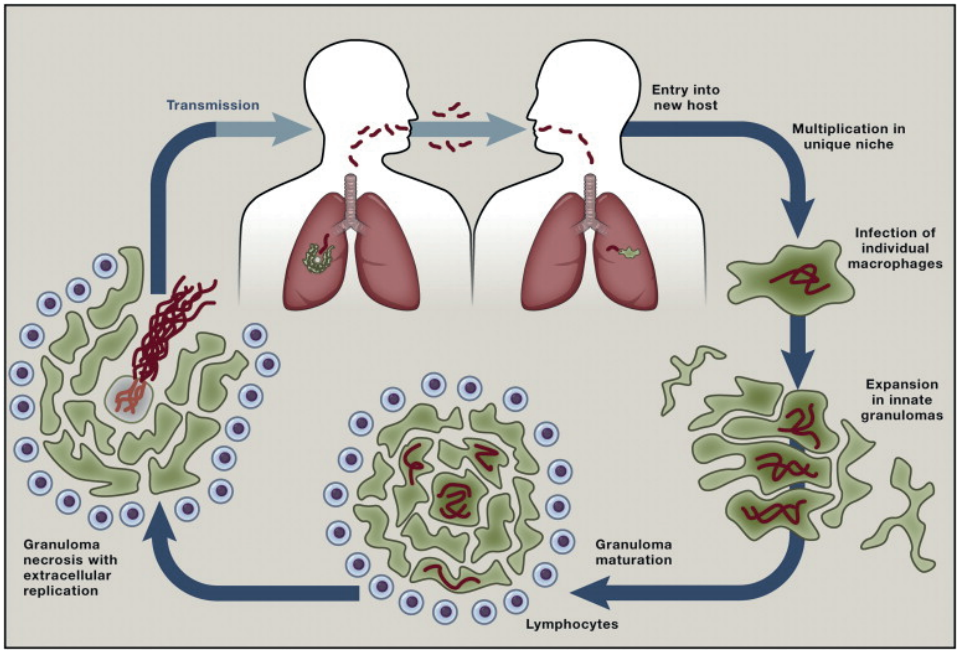

Figure 1. The infection cycle of *M. tuberculosis*

Infection starts when an infected individual coughs mycobacterium-containing aerosol particles that are subsequently inhaled by a new host and hereby are able to reach the lower respiratory tract. At the site of infection, recruited macrophages become infected and subsequently transport bacteria to deeper tissues. After new rounds of macrophage recruitment, the granuloma is formed, containing also other types of immune cells. In the early stages, the infection expands as mycobacteria successfully infect also newly recruited macrophages. After the first multiplication stage, the adaptive immune system is activated and the infection is stabilized. Usually, the immune system can restrict bacterial growth and further transmission. However, in some cases, the center of the infected granuloma can become necrotic, supporting bacterial growth and transmission of the tubercle bacilli to the next host. Figure copied from (Cambier *et al.*, 2014).

called ESX-1 to ESX-5, which perform distinct functions. Out of these five systems, three have been found to be functional in *M. tuberculosis* and to secrete substrates, namely ESX-1, ESX-3 and ESX-5. ESX-1, the first described system, is, as already mentioned, important for pathogenic mycobacteria to escape out the phagosomal compartment. The importance of T7SSs in pathogenicity is emphasized by the lack of ESX-1 in the live attenuated vaccine strain BCG (van der Wel *et al.*, 2007). ESX-3 has been implicated in the uptake of iron and zinc (Siegrist *et al.*, 2009, Serafini *et al.*, 2009). ESX-5, the most recently evolved system, is also essential for growth of *M. tuberculosis* and has been shown to be involved in the uptake of nutrients. The role in nutrient transport is probably

through ESX-5 substrates that are embedded in the OM, where they act as selective nutrient uptake pores (Ates *et al.*, 2015, Wang *et al.*, 2020, Korycka-Machala *et al.*, 2020).

Mycobacterial T7SSs are defined by a set of five conserved membrane components, two cytosolic components and a suite of substrates (Houben *et al.*, 2014). Four of the five membrane components, named EccB, EccC, EccD and EccE assemble into a large nanomachine that is involved in translocating substrates across the mycobacterial inner membrane (Houben *et al.*, 2012). MycP, the fifth conserved membrane component, interacts loosely with the membrane complex and is involved in complex stabilization (van Winden *et al.*, 2016). Each system secretes its own substrates that belong to four different protein classes, called Esx, PE, PPE and Esp. All these different substrates share several structural features and motifs.

SCOPE OF THIS THESIS

The aim of this thesis was to investigate the structural and functional characterization of the mycobacterial T7SS machinery.

In **Chapter 2** of this thesis, we discuss the current knowledge on mycobacterial T7SSs, with a focus on describing recent functional and structural advancements.

In **Chapter 3,** we describe our analysis of the general architecture of the T7SS membrane spanning machinery. For this, we reconstituted an ESX-5 system of *Mycobacterium xenopi* in the nonpathogenic strain *Mycobacterium smegmatis*. The system was properly assembled, functional and relatively overexpressed. The membrane complex of this system was purified and analyzed through negative stain electron microscopy and a suite of biochemical and biophysical tools. We described a large hexameric membrane complex that spans only the inner membrane, with highly flexible EccC ATPase subunits. Through multiple localization and identification methods, we inferred the location of individual subunits inside this complex.

In **Chapter 4,** we present our study on the species-specific recognition of PE_PGRS substrates by ESX-5 systems of different species. By creating chimeric constructs between *M. marinum* and *M. tuberculosis* $EccC_5$, we found that secretion of the ESX-5 dependent PE_PGRS proteins is dependent on the linker2 region of $EccC_5$, which connects the first and second nucleotide binding domain of the ATPase. Linker2 is considerably extended in ESX-5, the most recently evolved T7SS, which is also the only

system responsible for PE_PGRS secretion. From these results we proposed that this linker domain might be a substrate recognition site for this group of substrates.

In **Chapter 5** of this thesis, we describe our work on MycP. We could show that the MycP protease functions in close proximity to the EccB component within the T7SS membrane complex. While MycP has been shown to transiently interact with the membrane complex, its direct interacting partner remained unknown. By mimicking a naturally occurring fusion between EccB and MycP, seen in *Bifidobacterium dentium*, we showed that an $EccB_5$-$MycP_5$ fusion protein is able to functionally complement both an *eccB*$_5$ and a *mycP*$_5$ mutant in *M. marinum* and in the reconstituted ESX-5 system of *M. xenopi*, expressed in *M. smegmatis*. Additionally, this fusion protein could be stably and specifically co-purified together with the membrane complex, strongly indicating that MycP functions as a stable component of the T7SS membrane complex.

In **Chapter 6**, we set out to identify all components that are required for ESX-5 secretion and membrane complex assembly. We took advantage of the functionally reconstituted and plasmid encoded ESX-5 system of *M. xenopi* in *M. smegmatis* to produce a complete set of *esx-5* gene deletions. We could show that only the membrane components are required for successful membrane complex assembly. We also observed that Esx and PE/PPE proteins are mutually dependent on each other for secretion.

Finally, In **Chapter 7** we reflect on the work and results described in this thesis.

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